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Glyphosate pathways to modern diseases V: Amino acid analogue of glycine in diverse proteins

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Glyphosate, a synthetic amino acid and analogue of glycine, is the most widely used biocide on the planet. Its presence in food for human consumption and animal feed is ubiquitous. Epidemiological studies have revealed a strong correlation between the increasing incidence in the United States of a large number of chronic diseases and the increased use of glyphosate herbicide on corn, soy and wheat crops. Glyphosate, acting as a glycine analogue, may be mistakenly incorporated into peptides during protein synthesis. A deep search of the research literature has revealed a number of protein classes that depend on conserved glycine residues for proper function. Glycine, the smallest amino acid, has unique properties that support flexibility and the ability to anchor to the plasma membrane or the cytoskeleton. Glyphosate substitution for conserved glycines can easily explain a link with diabetes, obesity, asthma, chronic obstructive pulmonary disease (COPD), pulmonary edema, adrenal insufficiency, hypothyroidism, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, prion diseases, lupus, mitochondrial disease, non-Hodgkin's lymphoma, neural tube defects, infertility, hypertension, glaucoma, osteoporosis, fatty liver disease and kidney failure. The correlation data together with the direct biological evidence make a compelling case for glyphosate action as a glycine analogue to account for much of glyphosate's toxicity. Glufosinate, an analogue of glutamate, likely exhibits an analogous toxicity mechanism. There is an urgent need to find an effective and economical way to grow crops without the use of glyphosate and glufosinate as herbicides.

1. INTRODUCTION

While it might be expected that fidelity is always perfect in mapping from the DNA triple code to the specific amino acid it codes for, multiple studies have shown that this is not the case [1–5]. In addition to coding errors leading to substitution of another core amino acid, there exist hundreds of non-protein amino acids that could be substituted, some of which occur naturally in plants [1, 2]. Others are produced as oxidation products of the original amino acids [3]. In inflammatory conditions such as Alzheimer's disease, atherosclerosis and cataract generation, accumulation of oxidized proteins as components of lipofuscin are believed to contribute to the disease process [6]. Remarkably, oxidized amino acids can be directly incorporated into protein chains through protein synthesis [4]. These damaged peptides cannot be repaired except through complete enzymatic hydrolysis, and their accumulation with aging is believed to disrupt cellular functions.

Finally, and most significantly, multiple *synthetically* produced amino acids, close structural analogues of natural amino acids, can be mistakenly incorporated into peptides [7, 3]. There are 20 unique aminoacyl-tRNA synthetases in the ribosomal system, each of which specifically recognizes one amino acid, according to the DNA code. Ominously, there does not appear to be any proof-reading mechanism for the ribosomal system. Once an amino acid analogue fools the recognition process, there is no mechanism to abort translation and discard an erroneously produced peptide sequence [4]. A direct quote from Rodgers et al. [4] makes this very clear: "Certain structural analogues of the protein amino acids can escape detection by the cellular machinery for protein synthesis and become misincorporated into the growing polypeptide chain of proteins to generate non-native proteins." Glyphosate is a glycine molecule with a methyl-phosphonyl group bound to the nitrogen atom. As an analogue of glycine, it can be expected to displace glycine at random points in the protein synthesis process, with unknown consequences.

Godballe et al. describe in their 2011 paper how glycine can be used to construct synthetic molecules

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having functionality resembling the activities of cationic antimicrobial peptides [8]. A reactive side chain is attached to the nitrogen of glycine, and such units can be assembled into "peptoid" chains that in many ways resemble peptide chains, except that they are highly resistant to proteolysis. This is presumed to be beneficial because it allows the antimicrobial agent to survive longer in the tissues. These authors remarked: "N-substituted glycines can be viewed as amino acids, where the side chain is attached to the amine nitrogen instead of the α-carbon, and oligomers of these building blocks are called α-peptoids."

Glyphosate, is in fact, an N-substituted glycine; i.e., a peptoid unit. If glyphosate is misincorporated into a peptide under construction, it could interfere with the disassembly of the defective peptide, leading to the accumulation of undegraded short peptide chains with unknown consequences in the blood or in cells harbouring such defective proteins. It is intriguing and suggestive that phosphonyl groups are attractive as a component of designer peptides that inhibit proteases [9] and of potential insecticides that work by inhibiting protein degradation [10].

There is considerable evidence that glyphosate's biological effects are due in part to its action as a glycine analogue. Glyphosate disrupts chlorophyll synthesis in plants, likely due in (large) part to its inhibition of δ-aminolevulinic acid (ALA) synthesis, the rate-limiting step in the synthesis of the core pyrrole ring. It has been proposed that this may be a major factor, besides disruption of the shikimate pathway, in its toxicity to plants [11]. Its action as a glycine analogue likely causes competitive inhibition of ALA synthase from glycine and succinyl coenzyme A. Glyphosate has been shown to activate NMDA receptors in rat hippocampus [12], and this has been proposed to be in part due to glyphosate's ability to act as a ligand in place of glycine, in addition to glutamate (as the other ligand), whose overexpression is induced by glyphosate [13]. Both glyphosate and its metabolite aminomethylphosphonic acid (AMPA) can inhibit the growth of some tumour cells, likely by suppressing glycine synthesis [17].

If glyphosate substitutes for glycine in peptide sequences under construction, the results are likely to be catastrophic at multiple levels. The evidence that glyphosate interferes with glycine's rôles as a receptor ligand and as a substrate, and also suppresses glycine synthesis, implies that glyphosate could be taken up instead of glycine and subsequently incorporated into a peptide during protein synthesis. Several examples already exist of non-coding amino acids causing harm through misincorporation into peptides. For example, a natural non-coding amino acid analogue of proline, azetidine-2-carboxylic acid (Aze), is linked to multiple sclerosis due to its ability to displace proline in peptides [14]. Similarly, L-canavanine, a natural non-coding analogue of L-arginine, is a toxin stored in the seeds of certain plants [15, 16]. β-N-methylamino-L-alanine (BMAA), a natural analogue of serine synthesized by cyanobacteria, is implicated in amyotrophic lateral sclerosis (ALS) and other neurological diseases [1]. A recent study of glyphosate's effects on the rhizosphere microbiome showed sharp increases in the expression of proteins involved with both protein synthesis and especially protein degradation, implying that multiple synthesized proteins were failing to fold properly and had to be disassembled and reconstructed [18].

In this paper, we present a review of the literature on diverse biologically important proteins that contain either glycine-rich regions or conserved/invariant glycine residues. The evidence supports the likelihood that multiple diseases and conditions currently on the rise may be caused by disruption of conserved glycine residues, often in ways that would be predicted on the basis of glyphosate's physical properties.

Glycine plays many important rôles in human physiology, as an inhibitory neurotransmitter, as substrate for the biosyntheses of glutathione, haem, creatine, nucleic acids and uric acid, and as a source for one-carbon metabolism via the glycine cleavage system (GCS) [19]. Glycine also plays an important rôle in metabolic regulation and as an antioxidant. Finally, and perhaps most importantly, glycine is a highly conserved residue in diverse proteins, due to its unique properties. Glycine is the smallest amino acid, having no side chains. It is especially important in proteins that require flexibility, in hinge regions, or for ion gates that must open and close under varying circumstances [20]. Glycine is achiral, such that it can adopt angles representative of either L- or D-amino acids. Glycine confers flexibility through its unique ability to adopt a wide range of main-chain dihedral angles [21]. Many highly conserved glycine residues have been found in various proteins, reflecting this need for flexibility and mobility. It has also been determined empirically that substitution of conserved glycines in the enzyme acylphosphatase causes an increased tendency to aggregate, and this may be an important consideration for protection from the amyloid formation linked to many neurological diseases [22].

Glycine plays a critical rôle in dimerization for a number of protein classes for which dimerization is an essential step towards activation. Glycine is also highly conserved as the terminal residue in certain peptides, where it often plays a crucial rôle by supporting binding to the plasma membrane via myristoylation [23]. In many cases, even conservative substitution of alanine for

glycine disrupts the enzyme's function due to conformational changes following steric hindrance or impaired myristoylation. Conserved glycine residues are often located at the enzyme active site, particularly in the GXY or YXG motifs: glycine provides flexibility necessary to accommodate presence or absence of the substrate [24].

As of 2011, glyphosate was the largest selling herbicide worldwide [25]. In a series of previous publications [26–29], we have discussed how glyphosate's known toxicological mechanisms can be causal in a large number of diseases whose incidence is going up in step with the steadily increasing use of glyphosate on core corn, soy and wheat crops in the USA. The correlations between glyphosate usage and the recent alarming increase in multiple modern diseases are stunning, as presented in [30]. These include obesity, diabetes, end stage renal disease, renal failure, autism, Alzheimer's disease, dementia, Parkinson's disease, multiple sclerosis, intestinal infection, inflammatory bowel disease, stroke, leukemia, thyroid cancer, liver cancer, bladder cancer, pancreatic cancer and kidney cancer. Another study, looking at both human and animal data, revealed a large number of disorders of the newborn that are increasing in step with glyphosate usage [31]. These include congenital heart disease, skin disorders, genitourinary disorders, blood disorders, metabolic disorders and lung conditions. Our previous papers have been able to explain some of the pathology linked to glyphosate, predominantly through its powerful chelating effects, its adverse effects on beneficial gut microbes, its interference with the supply of crucial nutrients (in many cases derived from the shikimate pathway), and its suppression of cytochrome P450 enzymes in the liver.

However, given the large number of diseases and conditions that are correlated with glyphosate usage, we suspect that there is something much more insidious and fundamental than chelation or enzyme suppression that is happening with glyphosate. The fact that it is a synthetic amino acid, an analogue of an amino acid that carries many important rôles in the function of proteins containing it, makes it conceivable that glyphosate substitution for glycine in peptides could cause a large number of adverse effects that would not otherwise be anticipated. This would explain how a single toxic agent can be responsible for so many modern diseases.

2. BIOACCUMULATION, METABOLIZATION AND REACTION PRODUCTS OF GLYPHOSATE

The ability of glyphosate to bioaccumulate and metabolize *in vivo* in animals was clearly demonstrated in a 1988 study by Howe et al. [32]. Table 1 below outlines some of the study's design features. Seven groups of rats received a single oral or intravenous (IV) ^{14}C-radiolabeled dose of glyphosate technical acid (N-phosphonomethyl glycine). Group 6 was preconditioned with unlabeled glyphosate at 10 mg kg^{-1} day^{-1} for 14 days before receiving a single radiolabeled dose. AMPA and N-methyl AMPA (MAMPA) were the main metabolites found in the excreta, as well as other metabolites and reaction products. The fact that the research team found 0.3% of the dose as radioactive CO_2 in the expired air from the animals' lungs, within 24 hours, demonstrated *in vivo* metabolism. Glyphosate was the primary radiolabeled material found in the urine and faeces; bioaccumulation was found in all tissues, glands and organs. Additional details can be found in previously published work [29].

Table 1. Glyphosate metabolism experimental design by Howe et al. [32].

Group No.	Dose/ mg kg^{-1}	Animals	Route	Duration/ days	Samples collected
1	10	3 males 3 females	Oral	7	Urine, faeces, expired air @ 6, 12, 24 h
2	10	3 males 3 females	Oral	7	Blood @ 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 120 and 168 h
7	10	3 males 3 females	IV	7	ditto
3	10	5 males 5 females	IV	7	Urine and faeces @ 6, 12, 24 h and daily thereafter; organs, tissues, carcass @ day 7
4	1000	5 males 5 females	Oral	7	ditto
5	10	5 males 5 females	Oral	7	ditto
6	10[a]	5 males 5 females	Oral	7	ditto

[a] Group 6 was preconditioned with unlabeled glyphosate at 10 mg kg^{-1} day^{-1} for 14 days before receiving a single radiolabeled dose.

Glyphosate metabolism by plants was also investigated by Dupont in 2007 [33]. Protection from the effects of glyphosate was achieved through genetic engineering of maize plants to induce excess synthesis of the enzyme glyphosate acetyltransferase (GAT). The modified gene, *gat4601*, produces the enzyme acetolactate synthase, which acetylates glyphosate, thus preventing herbicide activity and plant death. N-acetylglyphosate (N-acetyl-N-phosphonomethyl glycine) is another amino acid and glycine analogue that was found in animals by Monsanto.

Acetylation does not preclude glyphosate's incorporation as an amino acid. N-acetylglyphosate can be recycled back to glyphosate *in vivo* through deacetylation. This has been shown to occur in both goats [34] and chickens [35]. The metabolization of N-acetylglyphosate includes its decarboxylation to N-acetyl AMPA, and further metabolism to AMPA, as illustrated in Fig. 1. Radiolabeled metabolism of N-acetylglyphosate was investigated in chickens [35], using orally dosed laying hens. Sacrificed hens, eggs and excreta were analysed/assayed for total ^{14}C, glyphosate, AMPA, N-acetylglyphosate and N-acetyl AMPA residues. Results are shown in Table 2. The fact that nearly 12% of the reaction products in egg yolk were recovered in the pepsin digest, and over 3% in the protease digest, suggests that glyphosate is being incorporated into peptide chains. The ^{14}C radioactivity in the enzyme digests indicated that an additional glyphosate analogue had been extracted; however, low residue levels precluded further analysis.

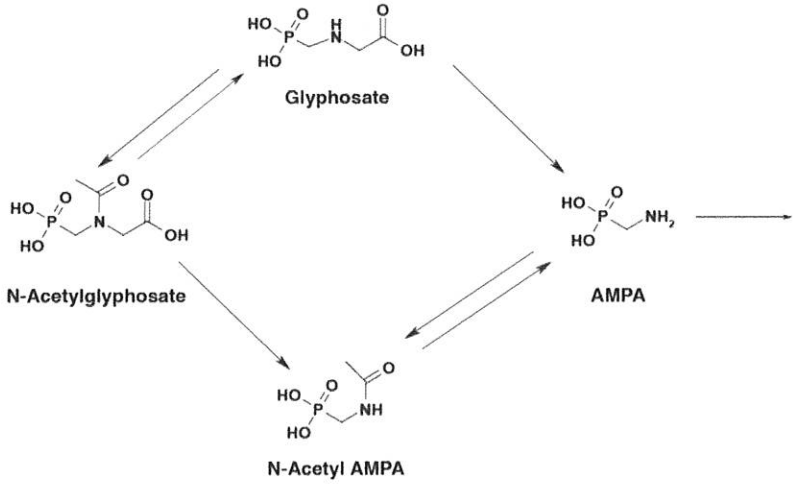


Figure 1. Glyphosate metabolism pathways.

Table 2. ^{14}C N-acetylglyphosate residues found in excreta, eggs and tissues (from Dupont, 2007 [35]).

Matrix	TRR[a]		Extracted		Unextracted	
	%dose	mg/kg eq[b]	%TRR	mg/kg eq	%TRR	mg/kg eq
Egg white	0.01	0.02	94.3	0.01	5.7	< 0.01
Egg yolk	0.04	0.34	81.5	0.19	18.5	0.04[c]
Whole egg		0.36[d]	na[e]		na	
Liver	0.05	0.51	95.6	0.48	4.4	0.02[c]
Muscle		0.04	87.5	0.03	12.5	<0.01
Abdominal fat		0.05	92.4	0.05	7.6	<0.01
Excreta		84.1		83.2	1.0	
Cage wash		5.9		na		na
Total recovery		90.2[f]		na		na

[a] Total radioactive residue.
[b] Equivalent value derived from liquid scintillation data.
[c] Egg yolk and liver post-extraction solids (PES) were subjected to enzyme digestion.
[d] Levels in reconstructed whole eggs calculated by summing (proportionally) residue levels in egg whites and yolks.
[e] Not applicable.
[f] Total recovery was derived by summing radioactivity in excreta, cage wash, egg yolks, egg whites and liver.

Remarkably, in Dupont's study on goats [34], muscle extraction yielded only 42% of the total reactivity before pepsin digest, and there was negligible additional recovery after both pepsin and protease digests. This suggests that glyphosate strongly inhibited the ability of proteases to break down the proteins, as 58% remained embedded and detectable only by its radioactive label. It was also noted that liver extraction recovery was 83% before pepsin digest and 6.9% additional recovery after digest. Kidney extraction was 97% before pepsin with an additional 4.6% recovery from the digest. Omental, renal and subcutaneous fat yielded 35, 94 and 92% recovery, respectively, before pepsin digest with an additional 28% recovery from omental fat only by pepsin digestion. Protease digestion in these tissues yielded insignificant levels of TRR recovery.

The Lowery/Dupont experiment with 5 laying hens studied the metabolism of ^{14}C-radiolabeled N-acetylglyphosate [35]. Birds were dosed by capsule twice per day for seven days with pure N-acetylglyphosate. Two radiolabeled substances were found in the chicken excreta and identified by HPLC, N-acetylglyphosate 82%) and glyphosate (0.8%). Residues of N-acetylglyphosate, AMPA, glyphosate and N-acetyl AMPA were identified in the liver, as well as six distinct radiolabeled residues in the abdominal fat. Sequential treatment with pepsin and protease enzymes of the total radioactive residues (TRR) remaining in the liver and egg yolk samples liberated additional radioactivity (4.1–14.7% TRR *in toto*), suggesting that glyphosate had been incorporated into the proteins.

A total of eight radiolabeled substances were found in actual muscle tissue, including: N-acetylglyphosate 25% (0.009 mg/kg); AMPA 17% (0.005 mg/kg); glyphosate 7.2% (0.002 mg/kg); N-acetyl AMPA 1.9% (0.001 mg/kg); and four additional metabolites representing 9% (0.003 mg/kg).

The highest bioaccumulated total radioactive residue in whole eggs was 0.36 mg/kg, occurring at seven days. Unmetabolized N-acetylglyphosate and metabolites of AMPA, glyphosate and N-acetyl AMPA were 0.16, 0.002, 0.014 and 0.003 mg/kg, respectively.

Egg whites and yolks were also examined individually. The results are summarized in Table 3.

Table 3. Distribution of total radioactive residues (TRR) of glyphosate metabolites and reaction products found in chicken eggs and tissues by liquid scintillation counting (LSC).[a]

Component	Composite egg white (day 1–7)		Composite egg yolk (day 1–7)		Liver		Composite muscle		Composite fat	
	% TRR	mg/kg eq	% TRR	mg/kg eq	% TRR	mg/kg eq	% TRR	mg/kg eq	% TRR	mg/kg eq
TRR (mg/kg eq)	na	0.010		0.229		0.505		0.033		0.057
initial extract	94	0.009	81	0.187	96	0.483	87	0.029	92	0.053
concentrated extract	94	0.009	80[b]	0.183	64[c]	0.322	87	0.029	92	0.053
AMPA	-[d]	-	0.91	0.002	6.7	0.034	17	0.005	11	0.007
glyphosate	11	0.001	5.7	0.013	16	0.084	7.2	0.002	39	0.023
N-acetyl-AMPA	4.3	<0.001	1.1	0.003	4.0	0.020	1.9	0.001	10	0.006
N-acetyl-glyphosate	41	0.004	68	0.157	64	0.323	25	0.009	23	0.014
minor unknowns	3.4	< 0.001	-	-	-	-	15[e]	0.006	1.4[f]	0.001
pepsin digest (PD)	na[g]	na	12	0.027	3.8	0.019	na	na	na	na
processed PD	na	na	4.3[h]	0.010	0.63[h]	0.003	na	na	na	na
protease digest	na	na	3.1[h]	0.007	0.27[h]	0.001	na	na	na	na
unextracted residues	5.7	0.001	3.8	0.008	0.36	0.002	13	0.004	7.6	0.004

[a] From Dupont, 2007 [35].
[b] Differences during processing reflect losses (1.5% TRR) incurred during concentration and/or sample clean-up for HPLC.
[c] Losses (32% TRR) during the process were attributed to non-selective adsorption to particulate matter in the concentrated extract.
[d] Not detected.
[e] Not applicable.
[f] Up to 4 components with no one component accounting for greater than 9% TRR (0.003 mg/kg eq).
[g] Up to 2 components with no one component accounting for greater than 0.7% TRR (< 0.001 mg/kg eq).
[h] Low levels of radioactivity in the concentrated digest precluded further characterization.

Glyphosate, like the canonical amino acids, is capable of chemical modification and metabolism *in vivo* [29]. The glyphosate amino acid analogues that are reaction products of these processes are shown in Fig. 2. Glyphosate can be acetylated, methylated, formylated and nitrosylated. Enzymatic deacetylation also recycles the acetylated molecule back to glyphosate. All of these modifications will impact the potential for glyphosate to be taken up by the cell and will change its reaction chemistry. For example, amino acid methylation generally makes the molecule both more water-soluble and more fat-soluble, as well as lowering the activation energy [36]. Fig. 3 shows metabolites of glyphosate that were found during Monsanto's experiments on rats. N-acetyl AMPA was identified by Dupont.

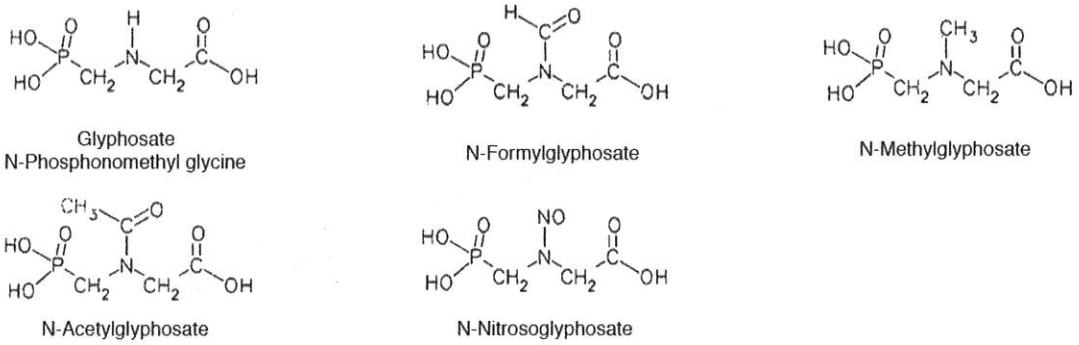
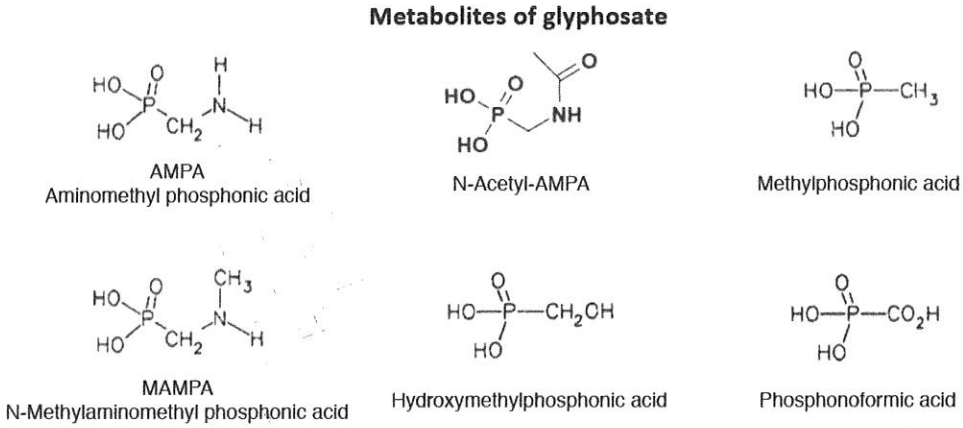


Figure 2. Glyphosate-derived amino acids identified by Monsanto exhibiting typical amino acid modifications.



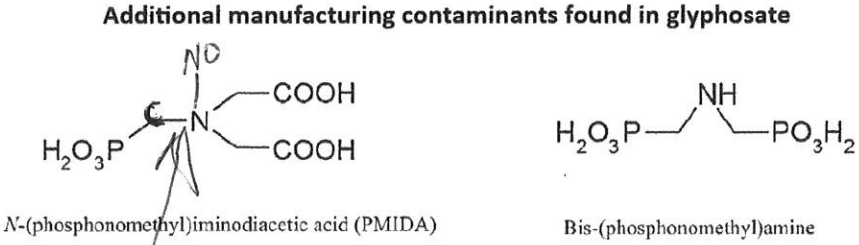


Figure 3. Metabolites and manufacturing contaminants of glyphosate.

3. DNA DAMAGE: CHROMATID DELETIONS AND ACHROMATIC LESIONS

One of Monsanto's early studies involved examining DNA damage in bone marrow of mice exposed to glyphosate [37]. An interesting finding was a substantial increase in the number of chromatid deletions and achromatic lesions observed following glyphosate exposure compared to controls (Table 4). Achromatic lesions (gaps) in chromatids are induced by endonucleases that play a rôle in the repair process. These gaps are a manifestation of unrejoined DNA double-strand breaks following endonuclease activity [38]. A possible explanation for the observed lesions involves impaired DNA repair mechanisms, particularly concerning the nucleotide guanine. 7,8-dihydro-8-oxoguanine (8-oxoG) is one of the most commonly formed oxidative lesions in DNA [39]. It is particularly destructive because it mispairs with adenine during replication, changing guanine:cytosine to thymine:adenine. Premutagenic lesions accumulate in mice that are defective in the gene coding the DNA glycosylase enzyme, OGG1, which excises 8-oxoguanine from DNA [40].

Table 4. Statistical analysis of data on chromatid deletions and achromatic lesions in rat bone marrow cells, performed only on data where the observed frequency for glyphosate treatment was higher than that of the solvent control.[a]

A. Chromatid deletions: observed frequencies[b]			
Sampling time	Control	Glyphosate (1g/kg)	p^c
12 h	0.0035	0.0087	0.26
24 h	0.0071	0.0142	0.26
B. Achromatic lesions: observed frequencies[b]			
Sampling time	Control	Glyphosate	p^c
6 h	0.0083	0.020	0.08
12 h	0.0052	0.016	0.08

[a] Table reproduced from Monsanto's 1983 report [37].
[b] No. of aberrations minus number of cells scored.
[c] Probability to be the same as the solvent control as determined by Student's *t* test.

Clustered DNA damage means multiple lesions in close proximity. In particular, it has been demonstrated experimentally that a lesion adjacent to an 8-oxoG is more resistant to the endonuclease-based repair process [41]. OGG1 has a conserved glycine at position G42 that plays an essential rôle in distinguishing 8-oxoG from guanine [42]. A hydrogen atom binds to the N7 atom of guanine during the formation of 8-oxoG, and this hydrogen atom is H-bonded to the carbonyl of the strictly conserved glycine residue in OGG1 to secure attachment. That is how it can recognize the oxidized form of guanine and distinguish it from the healthy, unoxidized molecule. Substitution of alanine for G42 disrupts the binding due to steric hindrance. With OGG1 impaired through glyphosate substitution for glycine, one can expect an accumulation of unrepaired 8-oxoG, leading to an increased frequency of clustered DNA damage and double strand breaks, and therefore of chromatid deletions and achromatic lesions, as observed by the Monsanto researchers. Mice with impaired OGG1 function develop increased adiposity, fatty liver disease and impaired glucose tolerance [39]. A defective version of this gene is linked to type-II diabetes in humans [43, 44].

4. METABOLIC AND SIGNALING DISORDERS

In this section we will examine several classes of enzymes that contain conserved glycines with essential rôles. We show that glyphosate substitution for glycine in hormone-sensitive lipase can explain an association between glyphosate and obesity, as well as adrenal insufficiency. The combination of protease inhibition and enhanced kinase activity can be predicted to cause excessive phosphorylation systemically. Phosphorylation is a widespread modification with profound effects on affected molecules, which can increase risk to both Alzheimer's disease and cancer. Pulmonary oedema induced by glyphosate can be explained through protein phosphatase inhibition.

The insulin receptor has conserved glycines that are necessary for its transport from the endoplasmic reticulum (ER) to the plasma membrane. Insufficient insulin receptor availability leads to hyperglycaemia and diabetes. Cytochrome c oxidase (COX) is the enzyme responsible for the final step of ATP synthesis in the mitochondrion. Substitutions for conserved glycines in COX severely impair oxidative phosphorylation. This can explain glyphosate's known toxicity to mitochondria. Kelch-like ECH-associated protein 1 (KEAP1) is a protein that regulates nuclear factor erythroid 2-related factor 2 (Nrf2)-like activity. It depends on a conserved glycine to prevent Nrf2 migration into the nucleus to activate multiple genes. Nrf2 overactivity can directly explain the beak deformities observed in chickadees fed sunflower seeds that were sprayed with glyphosate just before harvest. Nrf2 overactivity is also linked to fatty liver disease.

Hypothyroidism in the mother is a risk factor for autism in the child [45]. Disruption of conserved glycines in the pituitary gland can lead to insufficient release of thyroid-stimulating hormone. Conserved glycines also play a rôle in adrenocorticotropic hormone (ACTH) release, and ACTH deficiency has been linked to adrenal

insufficiency induced by glyphosate [46]. Both sulfate synthesis by endothelial nitric oxide synthase (eNOS) and the removal of sulfate from bioactive sulfated molecules can be predicted to be impaired upon glyphosate for glycine substitution at critical locations on eNOS and arylsulfatases. eNOS also depends on conserved glycines for nitric oxide synthesis. Impaired nitric oxide synthesis leads to hypertension.

4.1 Impaired cholesterol and fat metabolism

Lipases and esterases are an important group of enzymes that hydrolyse ester bonds. They contain a characteristic gly-xaa-ser-xaa-gly (GXSXG) motif; the essential active serine residue imparts the name "serine hydrolases" [47]. The hydrogen bond donated by the first glycine of the motif plays a critical rôle in the catalysis [48–50]. An especially interesting subclass of serine hydrolases are the hormone-sensitive lipases (HSLs) which, in humans, are responsible both for lipid hydrolysis and cholesterol ester hydrolysis [51]. HSLs respond to adrenalin, catecholamines and ACTH by initiating the release of fatty acids from adipose tissue as a source of fuel for the tissues [52]. HSLs are closely related to several bacterial proteins [53–55], and more distantly related to acetylcholinesterase and lipoprotein lipase. Hydrolase disruption leads to lipotoxic effects that can promote mitochondrial dysfunction, induce endoplasmic reticulum (ER) stress, induce inflammation, and compromise membrane function leading to apoptosis [56]. Impaired HSL function has been linked to obesity, atherosclerosis and type 2 diabetes [51].

In addition to the conserved GXSXG motif, members of the mammalian HSL class also contain the tetra-peptide histidyl-glycyl-glycyl-glycine (HGGG) motif in a conserved region described as an "oxyanion hole" [57, 58]. This is a critical element in the catalytic machinery of diverse proteolytic enzymes (notably serine protease and certain caspases), which stabilizes negative charge build-up in the substrate via hydrogen bonds.

Monsanto's chronic studies in mice and rats cited in our previous work [29] found considerable tissue destruction by glyphosate in the pituitary, thyroid, thalamus, testes and adrenal glands, as well as major organs. A 1990 study by Stout and Rueker revealed significant cortical adenomas, benign and metastatic pheochromocytomas and ganglioneuromas in male and female animals. A 1983 Knezevich and Hogan chronic study of glyphosate in mice revealed lymphoreticular tumours that "tended to be more frequent in treated animals, particularly the females." It revealed cortical cell adenoma and lymphoblastic lymphosarcoma of the adrenals.

A previous 1982 chronic study in rats by Lankas and Hogan also showed neoplastic phenomena in the adrenals, including reticulum cell sarcoma, pheochromocytoma, cortical adenomas and malignant lymphoma of the adrenals particularly in the female animals. "Pheochromocytoma of the adrenals was the second most common tumour found among male animals. Most frequent neoplastic changes of glands was seen in the pituitary gland which was highest in females" [59].

HSLs play an essential rôle in the adrenal glands as a first step in adrenal hormone synthesis from cholesterol [60]. The glyphosate-containing herbicide Roundup has been shown experimentally to severely impair adrenal hormone synthesis [46]. A glyphosate substitution for glycine in the GXSXG motif and/or the HGGG motif would disrupt protein function. This would also explain a link between glyphosate and obesity, due to impaired release of stored fats. The correlation between Roundup use on corn and soy crops and obesity in the USA as determined by data from the Centers for Disease Control (CDC) is very strong ($R = 0.96$, $P = 2 \times 10^{-8}$) [30].

4.2 Protease inhibition

Because excess expression of metalloproteinases is implicated in metastatic cancer, there is considerable interest in developing compounds that can inhibit protease activity [61]. Much effort has gone into developing protease inhibitors based on a phosphonyl moiety [9, 62]. The discovery of very potent irreversible inhibitors based on phosphonyl fluoride led to their use in *in vitro* studies, but they are highly unsuitable for therapeutic inhibition because they react with acetylcholinesterase, making them extremely toxic. Glyphosate, like phosphonyl fluoride compounds, has also been shown to inhibit acetylcholinesterase [63].

As a consequence of the toxicity of phosphonyl fluoride-based protease inhibitors, there has been a focus shift towards the concept of *peptidyl* phosphonate esters, because these can be hydrolysed, and because they can be designed to be specific to a narrow class of proteases. The attached polypeptide chain can be tuned to match the specificity of the target enzyme. Their mechanism of action is complex, but it involves a stable tetravalent phosphonylated derivative where one of the phosphonate oxygens is extended into an oxyanion hole (details can be found in [9] in the section beginning on p. 90). It can be expected that glyphosate's phosphonyl group might have a similar effect and, because of glyphosate insertion into a large number of different peptide sequences, the consequence of inhibition of multiple proteases by various glyphosate-containing short peptide chains, with unpredictable outcomes, can be expected.

4.3 Protein kinases, cancer and Alzheimer's disease

The human genome contains about 518 putative protein kinase genes, which constitute about 2% of all human genes [64]. Protein kinases contain a glycine-rich domain in the vicinity of the ATP-binding lysine residue in the N-terminal domain. The glycine-rich loop anchors the phosphate of ATP in a cleft just below the loop, and the nearby positively charged conserved lysine secures the nucleotide in place [65]. Protein kinase CK2 is a highly versatile molecule, able to phosphorylate more than 160 substrates on serine, threonine and tyrosine, using both ATP and GTP as phosphate donors [66]. It is involved in signal transduction and cell cycle regulation, cell proliferation and oncogenesis. A conserved region contains a glycine-rich loop (GXGXXG) that is also found in other protein kinases [67]. A model has proposed that the GXGXXG residues form an elbow around the nucleotide [68]. The second glycine, G48, is conserved in 99% of protein kinases, and it plays a fundamental rôle. Its replacement by negatively charged residues gives rise to mutants with improved kinetic properties for the peptide substrates. Insertion of a negatively charged residue favours faster release of ADP from the ATP pocket, leading to increased activity. It can be expected that glyphosate substituting for any of the conserved glycines in protein kinases, but especially G48, will *increase* protein activity.

Cyclin-dependent kinases (CDKs) are central to control of eukaryotic cell division. Their activity is regulated through phosphorylation and dephosphorylation of conserved threonine and tyrsosine residues [69]. GEGTYG is a highly conserved motif in CDK1, CDK2, CDK3, CDK5 and CDK10 [70]. This motif is referred to as the "G-loop," and the adjacent glycines are essential for maintaining the flexibility to control activation/inactivation by phosphorylation of the intervening threonine and tyrosine in the sequence GTYG. All the CDKs except CDK7 maintain the motif GXGXXG.

It has been suggested that overactivity of protein kinase CK2 plays an important rôle in cancer [71]: CK2 overexpression protects cellular proteins from caspase action and subsequent apoptosis. This leads to the transformation to a tumorigenic form supporting survival and proliferation. Imatinib (Gleevec) is a remarkably effective tyrosine kinase inhibitor used in chemotherapy to treat patients with leukaemia and breast cancer [72]. Many other drugs based on suppression of protein phosphorylation are under development [73].

Glycogen synthase kinase 3 (GSK3) is a constitutively active, proline-directed serine/threonine kinase, also containing a highly conserved glycine-rich N-terminus [74]. Its overexpression has been linked to Alzheimer's disease [75]. Overexpression of GSK3 can result in the hyperphosphorylation of tau, memory impairment, the increased production of β-amyloid (Aβ) and in the inflammatory response. GSK3 also reduces acetylcholine synthesis, and cholinergic deficit is a feature of Alzheimer's disease [76]. GSK3 also mediates apoptosis, which will promote the loss of neurons.

4.4 Insulin receptor activity and diabetes

The insulin receptor (IR) is a transmembrane tyrosine kinase receptor activated by both insulin and the insulin-like growth factors IGF-I and IGF-II. Defective IR activity can lead to type 2 diabetes [77], which has reached epidemic proportions throughout the industrialized world. The incidence of diabetes has been going up over time in the USA exactly in step with the increased use of glyphosate on core crops [30]. Knockout studies on mice, in which the insulin receptor of the α-cells of the pancreas were impaired, demonstrated that glucagon release is regulated by these receptors and, when they are dysfunctional, the mice display hyperglucagonaemia, hyperglycaemia and glucose intolerance [78]. A significant incidence of pancreatic islet cell tumours were reported in Monsanto studies in 1981 and 1990 (data shown in [29]).

A loosely conserved motif in two families of receptor tyrosine kinases, insulin receptors and epidermal growth factor receptors is characterized by a central glycine residue that allows for a turn in the secondary structure of the protein [79]. This glycine residue has an upstream α-helix and a downstream β-sheet. Receptors for insulin and epidermal growth factor both contain at least 8 repeats of this motif. The glycine-centred motif in the IR is thus very important in determining its three-dimensional structure [80]. A patient with leprechaunism, a genetic syndrome associated with extreme insulin resistance, had two mutations in the gene for IR, one of which was a glycine in this conserved loop [81]. Arginine was substituted for gly366 in the first repeat of the loop, and alanine displaced a conserved hydrophobic valine residue. Both mutations impair post-translational processing and intracellular transport of the receptors to the plasma membrane. Most likely, these two mutations inhibit the folding of the proreceptor into its normal conformation [80]. This results in its retention within the ER, and therefore post-translational processing steps in the Golgi apparatus are blocked. The result is a great reduction in the number of receptors that are transported to the plasma membrane and, therefore, impaired glucose uptake.

4.5 Cytochrome c oxidase

Glyphosate has been shown to disrupt oxidative phosphorylation in mitochondria, although this effect required dosages that were much higher than would be expected in realistic physiological situations [82]. Glyphosate in combination with surfactants has been shown to cause mitochondrial damage and induce apoptosis and necrosis [83]. It is possible that glyphosate induces toxicity to mitochondria through an effect on cytochrome c oxidase (COX), and that the surfactants enable glyphosate's entry into the cell and the mitochondria, greatly increasing its toxic effects on the latter [84].

This would be especially so for the salts and esters of glyphosate, which are more soluble than glyphosate technical acid (N-phosphonomethylglycine), which was used in Monsanto's chronic animal studies. It is interesting to note that the active principles actually used in Roundup glyphosate-based herbicide formulations in real-world applications are not solely the technical acid but rather the far more soluble salts and esters of glyphosate; i.e., potassium glyphosate, sodium glyphosate, ammonium glyphosate and the popular isopropylamine glyphosate. These formulations have been shown to be orders of magnitude more toxic than glyphosate in isolation [85].

COX catalyses the one-electron oxidation of four molecules of reduced cytochrome c and the four-electron reduction of oxygen to water. It is an essential component of the oxidative phosphorylation pathway in mitochondria that produces adenosine triphosphate (ATP), the "energy currency" of cells. Subunit II of COX contains a Cu_A redox centre, serves as a binding partner for cytochrome c, and as a participant in the electron transfer process [86]. Subunit II has a highly conserved glycine residue at the active site [87–89]. A mutant form of COX in *Rhodobacter sphaeroides* involving a substitution of valine for the conserved gly283 resulted in a complete block of access of oxygen to the active site [88]. Similarly, conversion of a conserved glycine in subunit II's active site to arginine in a yeast strain resulted in respiration deficiency [89]. A structural model of the redox center of subunit II includes *two* conserved glycines at positions 219 and 226, in close proximity to conserved amino acids that act as ligands to the Cu_A redox site and a glutamic acid residue implicated in cytochrome c binding, as schematized in Fig. 4 [90]. Obviously, substitution of glyphosate for glycine in either of these conserved sites would almost certainly harm enzyme function, leading to both impaired energy generation and oxidative damage. Glyphosate is also a strong chelator of copper, having a higher metal chelate formation constant—11.93—compared to its affinity for manganese (5.47), zinc (8.74) and calcium (3.25).

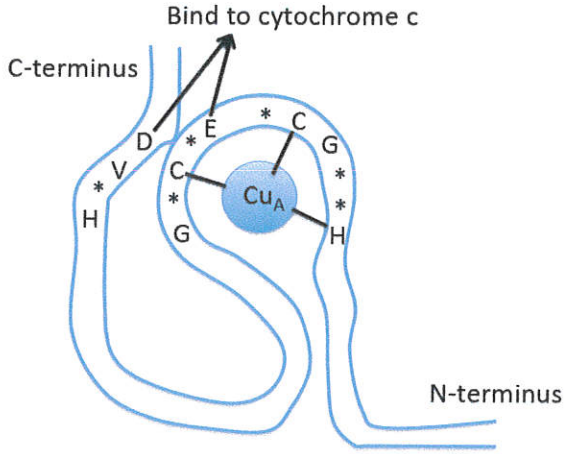


Figure 4. Schematic of structure of subunit II of cytochrome c oxidase (COX). Non-conserved amino acids are indicated by *. Adapted from Holm et al. (1987) [90].

4.6 Nrf2, KEAP1, fatty liver disease and bird beak deformities

Nrf2 is a leucine zipper protein that protects against oxidative damage due to an inflammatory response following various environmental triggers [91]. Interestingly, tumour cells often overexpress Nrf2, and this allows them to thrive in the face of severe oxidative stress [92–96]. High levels of Nrf2 activity cause chemotherapeutic resistance and correlate with a poor prognosis [94, 97].

Remarkably, although Nrf2 is cytoprotective, unregulated expression of Nrf2 is lethal in mice. Nrf2 is constitutively expressed, and KEAP1 is a cytoplasmic protein that regulates Nrf2 expression by binding to it to prevent its migration into the nucleus, thus enabling ubiquitination and subsequent degradation [98, 99]. Mice engineered to be KEAP1 deficient died postnatally, probably from malnutrition due to hyperkeratosis obstructing the oesophagus and forestomach [100]. The issue is that Nrf2 activates squamous epithelial cells to overproduce keratin, and a thickened oesophagus eventually becomes completely blocked.

KEAP1 maintains a cytoplasmic anchor through scaffolding with the cytoskeleton [98, 101]. The binding process depends upon a conserved region of the protein containing a sequence of two glycine residues (double glycine repeat, DGR). KEAP1 acts as a sensor for electrophilic and oxidative stresses to maintain an appropriate amount of Nrf2 activity. KEAP1 responds to oxidative stress through oxidation of sulfhydryl groups in conserved cysteine residues, and this causes it to release Nrf2, permitting its survival and entry into the nucleus, where it activates many phase 2 antioxidant defences

[102]. Unregulated overactivation of Nrf2 due to impaired KEAP1 function can be expected to lead to hyperkeratosis.

A newly emerging disease termed "avian keratin disorder" has become widespread among birds in certain regions of North America, particularly the interior of Alaska [103, 104], around the Great Lakes [105, 106], and off the coast of California (where agricultural runoff is a suspected factor) [107]. High rates of crossed beaks and other malformations were first noted around the Great Lakes in the mid-1970s [103, 105, 106], which is when glyphosate was first introduced into agricultural practice.

Chickadees are the most affected species, and they are known to frequent bird feeders supplying sunflower seeds, which according to the USDA are primarily grown in California, Colorado, the Dakotas, Kansas, Nebraska, Minnesota and Texas. Glyphosate is used in pre-planting, burndown, staging and preharvest dessication on sunflowers and specifically recommended to reduce crop loss due to feeding by wild blackbirds [108]. Frequent sightings of blackbirds with deformed beaks were first reported in 1979 [109].

A detailed study of potential toxic exposures to black-capped chickadees in Alaska, which investigated multiple toxic metals, organochlorine pesticides, polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDFs), was unable to identify any obvious exposure–disease relationship and, furthermore, the authors admitted that there was no known link between any of these chemicals and hyperkeratosis [104]. Notably, glyphosate was not studied. Avian keratin disorder is not present at birth, but rather develops over time and is most common among adult birds. Some physically examined birds revealed a systemic hyperkeratosis not limited to the beak. The most plausible explanation is that glyphosate substitutes for glycine in KEAP1, causing constitutive expression of Nrf2 leading to hyperkeratosis.

Non-alcoholic fatty liver disease (NAFL) has become an epidemic worldwide in recent years [110]. From 10 to 20% of patients with NAFL eventually develop non-alcoholic steatohepatitis (NASH), cirrhosis, end-stage liver disease, and hepatocellular carcinoma [111]. Mallory–Denk bodies (MDBs) are cytoplasmic inclusions associated with both alcoholic and non-alcoholic steatohepatitis [112]. These bodies are enriched in keratin, which is overexpressed through enhanced Nrf2 expression [111].

Non-melanoma skin cancer is the most common form of cancer among Caucasians [113]. Lankas and Hogan (1982) found sebaceous gland adenoma, and basosquamous cell tumour of the skin as well as fibrosarcoma, fibromas, neurofibrosarcoma, osteogenic sarcoma and mixed malignant tumour of the subcutaneous tissue associated with glyphosate residue ingestion by male rats during a 26-month study [59, 29]. Hyperkeratosis is a common feature of non-melanoma skin cancer [114]. Laryngeal keratosis is a risk factor for subsequent carcinoma [115]. Hyperkeratosis was observed in 2% of oesophageal biopsies performed on 1845 patients, and was linked to invasive squamous carcinoma of the oral cavity/larynx [116].

4.7 Tyrosine phosphatase and systemic inflammation

Glycine is a component of multiple sequence motifs that are consistent patterns within various groups of protein phosphatases. One sequence that includes a GXG subsequence is found in tyrosine phosphatases [117]. Another unique sequence containing two glycines is found in serine/threonine phosphatases [118]. Several acid phosphatases contain the conserved sequence, RHG [119]. A long signature motif found in a family of glucose-6-phosphatases, as well as several acid phosphatases and lipid phosphatases, contains a conserved glycine residue near the middle of the conserved sequence [120].

Protein tyrosine phosphatases are a class of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins, and they generally have an anti-inflammatory rôle [121]. Tyrosine phosphatases play a very important rôle in the developing immune system, influencing the process of maturation of T-cells, as well as in the immune response in the adult [122]. Defective versions of a haematopoietically expressed cytoplasmic tyrosine phosphatase have been associated with multiple autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and type 1 diabetes [123–127]. T-cell protein tyrosine phosphatase (TCPTP), a negative regulator of JAK/STAT and multiple growth factor receptors, is highly expressed in haematopoietic tissues [128]. Defects in this gene have been linked to type 1 diabetes, rheumatoid arthritis and Crohn's disease through genome-wide association studies [124, 128, 129]. Substitution of proline or alanine for the conserved gly127 residue resulted in a 400-fold decrease in catalytic activity [130].

Studies on TCPTP-deficient mice have greatly enhanced our knowledge of this important protein [131–133, 128, 134, 135]. Homozygous TCPTP-deficient mice become ill and die by three to five weeks of age [131–133, 128]. They exhibit severe anaemia and infiltration of mononuclear cells into multiple tissues, along with a dramatic increase in expression of proinflammatory

cytokines systemically, including TNF-α, IFN-γ and IL-12 [133]. More specifically, inflammation of the synovial membrane and severe subchondral bone resorption of the knee were observed, along with significantly greater numbers of osteoclasts in the femur [123]. Heterozygous TCPTP-deficient mice respond with excess cytokine production and exaggerated gut inflammation to epithelial insults, inducing colitis [136].

Severe glyphosate-surfactant poisoning is manifested by gastroenteritis, respiratory disturbances, altered mental status, treatment-resistant hypotension, renal failure and shock [137]. The fatality rate ranges from 3 to 30%, and is mostly due to either pulmonary toxicity or renal failure. A case study from India specifically highlights pulmonary oedema following acute poisoning, along with a precipitous drop in blood pressure [138], probably due to loss of serum fluids into the abdominal and pleural cavities.

A paper from 1990 by Martinez et al. compared the effects of an oral dose of Roundup on rats to intratracheal installations [139]. The oral dose induced pulmonary oedema 6 h later, along with bloodstained weeping from the nose, diarrhoea, distended gastrointestinal (GI) tract, and ascites, suggestive of hypovolemic shock. The intratracheal instillations were much more toxic at much lower dose levels. A dose of 0.1 mg/animal caused 80% mortality, and 0.2 mg/animal gave 100% mortality. Pathological examination found haemorrhaging and congestion in the lungs.

Protein tyrosine phosphatase plays a crucial rôle in protection from pulmonary oedema by maintaining barrier function following an inflammatory episode [140, 141]. It is conceivable that, following an acute inflammatory response to glyphosate poisoning, glyphosate is taken up by cells and incorporated into newly synthesized tyrosine phosphatase, disabling its effectiveness. However, glyphosate would likely inhibit these phosphatases even in the absence of direct incorporation into the peptide chain. An investigation into 15 different synthetic compounds, all of which contained a phosphonyl group, demonstrated their effectiveness in inhibiting both tyrosine and serine–threonine phosphatases [142].

Chronic obstructive pulmonary disease (COPD) is the fourth largest cause of death in the USA. It has been linked directly to overexuberant kinase-based signaling cascades [143]. Enhanced kinase activity combined with impaired ability to turn off the signal through dephosphorylation, both of which can be explained by glyphosate interference, can easily account for such a pathology.

Monsanto's sealed documents filed with the US EPA for the registration of glyphosate technical acid show that glyphosate has adverse effects on the lungs of animals. We previously reported tumours found in the lungs of test animals [29]. The study authors also noted many non-neoplastic microscopic findings. In 1981, Lankas and Hogan reported that the more common findings were changes in the kidneys and lungs. The lungs of many of the rats had "changes associated with chronic respiratory disease such as the presence of peribronchial and perivascular mononuclear cells and foci of macrophages in alveoli." In addition, some of the physical symptoms included nasal discharge, excessive lacrimation and rales (abnormal crackling noises) caused by disease and congestion of the lungs. Tumours of lungs were also found and included reticulum cell sarcoma, malignant lymphoma, adenocarcinoma and carcinomas.

Monsanto's studies found that radiolabeled carbon in glyphosate was able to be recovered in the exhaled breath of rats [29]. *Pseudomonas aeruginosa* is among the very few microbial species that are known to be able to metabolize glyphosate and use it as a source of phosphorus [144]. *P. aeruginosa* infection has been linked to COPD [145]. Glyphosate is known to disrupt bacterial homeostasis leading to an overgrowth of resistant pathogens; it was found by the USGS to be present in the atmosphere [146]; thus inhalation of the compound (not just ingestion) would also harm the lung.

4.8 Hypothyroidism due to impaired thyroid-stimulating hormone activity

In [45], it was proposed that impaired activity of manganese-dependent protein phosphatase 1 (PP1) could explain a link between autism and maternal hypothyroidism, due to a dependency on PP1 for the pituitary to release thyroid-stimulating hormone (TSH). In that paper, it was argued that glyphosate chelation of manganese might severely decrease manganese bioavailability. This argument was supported by the extremely low serum levels of manganese found in dairy cows exposed to GM Roundup-Ready feed [147].

However, we have already seen that phosphatases contain conserved glycine motifs that are essential for their proper functioning. Another distinct possibility is that glyphosate substitutes for glycine directly in the conserved CAGYC region of the β-subunit of TSH itself. A rare mutation where the central glycine in CAGYC is replaced by arginine in an autosomally recessive trait results in cretinism (mental and growth retardation) [148]. This single mutation leads to the synthesis of a defective form of the β-subunit of TSH, which renders it unable to associate with an α-subunit. This results in severe systemic deficiency of TSH and hypothyroidism. It is plausible that a similar disruption of adrenal stimulation occurs because of glyphosate substitution for a conserved glycine in ACTH [149]. A homozygous